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# Antioxidant activity, ascorbic acid, phenolic compounds and sugars of wild and commercial *Tuberaria lignosa* samples: Effects of drying and oral preparation methods

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ABSTRACT

The antioxidant activity and phytochemical composition (ascorbic acid, free sugars and phenolic compounds) of decoctions and infusions of wild and commercial samples of *Tuberaria lignosa* (Sweet) Samp. Aerial parts were evaluated and compared. Among wild samples, the effects of the drying method (freeze or shade-drying) on those parameters were studied. Infusion of the freeze-dried wild sample gave the highest levels of sugars, while infusion of shade-dried wild sample and decoction of the freeze-dried sample presented higher ascorbic acid and phenolic compounds content (including ellagitannins and flavonoids) than the other samples. The last two samples also revealed higher antioxidant activity, in some cases even higher than Trolox. Decoctions gave lower amounts of disaccharides than infusions, which seemed to be hydrolysed, increasing the content of monosaccharides. Commercial samples showed the lowest content in phenolic compounds, mainly in ellagitannins and flavonoids, and also the lowest antioxidant activity. This work gives scientific evidence to the traditional medicinal uses of wild *Tuberaria lignosa*, highlighting the interest of its decoctions and infusions as a source of bioactive compounds and functional beverages.



## 1. Introduction

The adverse effects of oxidative stress on human health have become a serious issue. Under stress, the concentration of reactive oxygen species (ROS) (e.g., superoxide anion radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide) in our bodies is higher than the concentration of enzymatic antioxidants (e.g., superoxide dismutase, glutathione peroxidase and catalase) and non-enzymatic antioxidants (e.g., ascorbic acid, vitamin E and glutathione) (Ferreira & Abreu, 2007; Krishnaiah, Sarbatly, & Nithyanandam, 2011). This imbalance leads to damage to lipids, proteins, carbohydrates and DNA, and consequently induces degeneration, destruction and toxicity of various biomolecules (Halliwell, 1996; Valko et al., 2007). The development of many diseases, including atherosclerosis, cardiovascular disease, cataracts, rheumatoid arthritis, inflammatory disorders, anaemia, asthma, cancer, and Parkinson's and Alzheimer's diseases, as well as ageing, is connected with oxidative stress (Halliwell, 1996; Shekhawat, Payal, Singht, & Vijayvergia, 2010; Valko et al., 2007). One solution to this problem is to supplement the diet with antioxidant compounds that are contained in natural plant sources. These natural plant antioxidants can therefore serve as a type of preventive medicine (Krishnaiah et al., 2011).

The plant kingdom offers a wide range of natural antioxidants. However, little is known about the practical usefulness of most of them. Many herbal beverages, frequently used in folk medicine, have antioxidant and pharmacological properties linked with the presence of phenolic compounds, especially flavonoids (Cabrera, Artacho, & Giménez, 2006; Pinto, 2010). The recognition of herbal beverages as functional drinks might be related with the plant species from which they are prepared and with formulation or preparation methods (Henning et al., 2004; Milašienė et al., 2007).

In Portugal as well as in Spain, some of the most popular medicinal plants have been traditionally gathered for preparing herbal infusions or decoctions (Pardo de Santayana, Blanco, & Morales, 2005). Such is the case of *Tuberaria lignosa* (Sweet) Samp. (Fam. Cistaceae), a species native to western and southern Europe, which is mainly present in the western regions of the Iberian Peninsula (Castroviejo, 2005). It is one of the most quoted medicinal plants in the Northeastern Portuguese region where it is popularly known as "alcária" or "erva-loba" (Carvalho, 2010). In traditional folk medicine, different parts (roots and aerial parts) of this plant are

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used for treating various diseases and ailments, such as gastrointestinal disorders, heartburn, wounds, influenza, skin infections and warts due to its vulnerary, anti-inflammatory and anti-infectious properties (Bedoya, Abad, Sánchez-Palomino, Alcami, & Bermejo, 2010; Carvalho, 2010; Martín-Aragón, Benedí, & Villar, 1994; Novais, Santos, Mendes, & Pinto-Gomes, 2004). Flowers, leaves or whole plant are prepared fresh or shade-dried in medicinal infusions and decoctions (Carvalho, 2010; Novais et al., 2004). Commonly, infusions of leaves or inflorescences are used for hepato-depurative disorders (internal use) and decoctions of the whole plant for skin inflammations (external and topical use). However, in Portugal local healers and key informants avoid using the *T. lignosa* plant extracts in humans for long periods, so as to prevent, to a certain degree, toxicity and adverse effects, which can vary considerably according to the method of preparation, doses and physical condition of the individual (Carvalho, 2010).

In this study, the antioxidant activity and phytochemical composition (ascorbic acid, free sugars and phenolic compounds) of decoctions and infusions of wild and commercial samples of *T. lignosa* flowering aerial parts (e.g., basal leaves, stems and inflorescences) were evaluated and compared. Furthermore, among wild samples, the effects of the drying method (freeze or shade-drying) on those parameters were studied.

## 2. Materials and methods

### 2.1. Samples

*T. lignosa* Sweet Samp. (synonym of *Xolantha tuberaria* (L.) Gallego, Muñoz Garm & C. Navarro) is a perennial species in the rockrose family Cistaceae, native to western and southern Europe, occurring mainly in dry, stony sites of the western Mediterranean area. The leaves are in a rosette at the base of the plant and the yellow flowers are organised in relaxed determinate inflorescences (Castroviejo, 2005).

Wild samples of *T. lignosa* were collected in the flowering season in Miranda do Douro (Trás-os-Montes, north-eastern Portugal), considering the local medicinal uses as well as healers' and selected consumers' criteria, which are related to particular gathering sites, and requirements for safe herbal dosages forms, such as infusion and decoction. In the area of sample collection annual rainfall is about 600–800 mm, the average temperature $12.5 \geqslant T > 10$ °C and altitude 600–700 m above sea level (Agroconsultores, 1991). Samples were submitted to two different drying processes: (1) Freeze-drying (7750031 Free Zone 4.5, Labconco, Kansas City, MO) immediately after being collected; (2) shade-drying, being stored in a dark and dry place in cellophane or paper bags kept at room temperature (~21 °C and 50% relative humidity) for 30 days, simulating informants' general conditions of use. Voucher specimens were deposited in the Herbarium of the Escola Superior Agrária de Bragança (BRESA, ETBO55/2011).

Commercial samples were obtained in a local herbal shop, in Bragança (north-eastern Portugal) and were available as dried rosettes of leaves and inflorescences. Usually herbal shops order vegetal materials for processing and pack from local inhabitants of the villages, so that wild and commercial samples may have the same origin.

### 2.2. Preparation of decoctions and infusions

#### 2.2.1. Decoctions

Each sample (15 g, according to Portuguese informants' practices; Carvalho, 2010) was added to 500 mL of distiled water, heated on a heating plate (VELP Scientific, Usmate, Italy) and boiled for 5 min. The mixture was left to stand at 25 °C for 5 min more, and then filtered under reduced pressure. The obtained decoctions were frozen and lyophilised.

#### 2.2.2. Infusions

Each sample (7.5 g, respecting local practices; Carvalho, 2010) was added to 500 mL of boiling distiled water and left to stand at 25 °C for 5 min, and then filtered under reduced pressure. The obtained infusions were frozen and lyophilised.

### 2.3. Standards and reagents

Acetonitrile (99.9%, HPLC grade) was from Lab-Scan (Lisbon, Portugal). Formic acid was purchased from Prolabo (VWR International, France). L-Ascorbic acid, sugar standards (D(−)-fructose, D(+)-glucose anhydrous, D(+)-raffinose pentahydrate, D(+) sucrose and D(+)trehalose) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma (St. Louis, MO). The phenolic compound standards (apigenin-6-*C*-glucoside, *p*-coumaric acid, ellagic acid, gallic acid, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, luteolin-6-*C*-glucoside, quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside) were from Extrasynthèse (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA). Water was treated in a Milli-Q water purification system.

### 2.4. In vitro antioxidant properties

#### 2.4.1. General

Lyophilised preparations of the decoctions and infusions were redissolved in water at a concentration of 2 mg/mL and further diluted to different concentrations to be submitted to distinct *in vitro* assays (Pinela, Barros, Carvalho, & Ferreira, 2011) to evaluate their antioxidant properties. The extract concentrations providing 50% of antioxidant activity or 0.5 of absorbance ($EC_{50}$) were calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene bleaching and TBARS assays) or absorbance at 690 nm (reducing power assay) against extract concentrations. Trolox was used as standard.

#### 2.4.2. DPPH radical-scavenging activity

This methodology was performed using an ELX800 Microplate Reader (Bio-Tek, Potton, UK). The reaction mixture in each one of the 96-wells consisted of one of the different solution concentrations (30 μL) and methanolic solution (270 μL) containing DPPH radicals ($6 \times 10^{-5}$ M). The mixture was left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation:

$$\mathrm{RSA}(\%) = [(A_{\mathrm{DPPH}} - A_S)/A_{\mathrm{DPPH}}] \times 100 \quad (1)$$

where $A_S$ is the absorbance of the solution when the sample extract has been added at a particular level, and $A_{\mathrm{DPPH}}$ is the absorbance of the DPPH solution.

#### 2.4.3. Reducing power

This methodology was performed using the microplate reader described above. The different solution concentrations (0.5 mL) were mixed with sodium phosphate buffer (200 mM, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 °C for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in 48-well plates, with deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm.

#### 2.4.4. Inhibition of β-carotene bleaching

A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution were pipetted into a round-bottom flask. After the chloroform was removed at 40 °C under vacuum, linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distiled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing different concentrations of the samples (0.2 mL). The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm in a Model 200 spectrophotometer (AnalytikJena, Jena, Germany). β-Carotene bleaching inhibition was calculated using the following equation:

$$(\text{Abs after2h of assay}/\text{initial Abs}) \times 100 \quad (2)$$

#### 2.4.5. Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)

Porcine (*Sus scrofa*) brains were obtained from official slaughtering animals, dissected, and homogenised with a Polytron in ice-cold Tris–HCl buffer (20 mM, pH 7.4), to produce a 1:2 (w/v) brain tissue homogenate which was centrifuged at 3000*g* for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with the different solution concentrations (0.2 mL) in the presence of $FeSO_4$ (10 μM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 mL), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 mL), and the mixture was then heated at 80 °C for 20 min. After centrifugation at 3000 *g* for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde (MDA)–TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula:

$$\text{Inhibition ratio}(\%) = [(A - B)/A] \times 100\% \quad (3)$$

where *A* and *B* were the absorbance of the control and the compound solution, respectively.

### 2.5. Phytochemical characterisation

#### 2.5.1. Vitamin C

Each lyophilised decoction/infusion (100 mg) was extracted with metaphosphoric acid (1%, 10 mL) for 45 min at room temperature and filtered through Whatman No. 4 filter paper. The filtrate (1 mL) was mixed with 2,6-dichloroindophenol (9 mL) and the absorbance was measured after 15 min at 515 nm (Pinela et al., 2011). Content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid ($6.0 \times 10^{-3}$–$1.0 \times 10^{-1}$ mg/mL), and the results were expressed as mg of ascorbic acid per grams of lyophilised decoction/infusion.

#### 2.5.2. Free sugars

Each decoction/infusion (1 mL) was filtered through 0.2-μm nylon filters from Whatman, and analysed by high-performance liquid chromatography coupled to a refractive index detector (HPLC-RI) as described by the authors (Pinela et al., 2011). The equipment consisted of an integrated system with a Smartline system 1000 pump (Knauer, Berlin, Germany), a Smartline Manager 5000 degasser, an AS-2057 auto-sampler (Jasco, Easton, MD) and a Smartline 2300 RI detector. Data were analysed using Clarity DataApex 2.4 Software. The chromatographic separation was achieved with a Eurospher 100-5 $NH_2$ column (4.6 × 250 mm, 5 mm; Knauer) operating at 35 °C in a 7971 R Grace oven. The mobile phase was acetonitrile/deionized water, 70:30 (v/v), at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards. For quantitative analysis, a calibration curve was obtained by injection of known concentrations (0.375–24 mg/mL) of different standard compounds: D(−)-fructose ($y = 1.04x$, $R^2 = 0.9999$), D(+)-glucose anhydrous ($y = 0.935x$, $R^2 = 0.9991$), D(+)-sucrose ($y = 1.087x$, $R^2 = 0.9999$), trehalose ($y = 0.991x$, $R^2 = 0.9999$) and raffinose ($y = 0.891x$, $R^2 = 0.9999$). The results were expressed as milligram per gram of lyophilised decoction/infusion.

#### 2.5.3. Phenolic compounds

Each lyophilised decoction/infusion (1 mg) was dissolved in water:methanol (80:20 v/v), filtered through 0.2-μm nylon filters from Whatman, and analysed by HPLC (Hewlett–Packard 1100 chromatograph, Agilent Technologies, Waldbronn, Germany) with a quaternary pump and a diode array detector (DAD) coupled to an HP ChemStation (Rev. A.05.04) data-processing station. A Spherisorb S3 ODS-2 $C_{18}$ (Waters, Dinslaken, Germany), 3 μm (4.6 mm × 150 mm) column thermostatted at 35 °C was used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was 10% B to 15% B over 5 min, 15–25% B over 5 min, 25–35% B over 10 min, isocratic 50% B for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyser that was controlled by Analyst 5.1 software. Zero grade air served as the nebuliser gas (30 psi) and turbo gas for solvent drying (400 °C, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupoles were set at unit resolution. The ion spray voltage was set at −4500 V in the negative mode. The MS detector was programm to perform a series of two consecutive scan modes: enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to obtain full scan spectra, to give an overview of all the ions in sample. Settings used were: declustering potential (DP) −450 V, entrance potential (EP) −6 V, collision energy (CE) −10 V. Spectra were recorded in negative ion mode between *m*/*z* 100 and 1500. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) of the previous experiment using the following parameters: DP −50 V, EP −6 V, CE −25 V, and collision energy spread (CES) 0 V.

The phenolic compounds present in the decoctions/infusions were characterised according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, calibration curves were prepared by injection of known concentrations (2.5–100 μg/mL) of different standard compounds: apigenin-6-*C*-glucoside ($y = 246.05 \times - 309.66$; $R^2 = 0.9994$); *p*-coumaric acid ($y = 321.99x + 98.308$; $R^2 = 0.9984$); ellagic acid ($y = 35.695x - 265.7$; $R^2 = 0.9991$) gallic acid ($y = 556.94x + 738.37$; $R^2 = 0.9968$); kaempferol-3-*O*-glucoside ($y = 190.75x - 36.158$; $R^2 = 1$); kaempferol-3-*O*-rutinoside ($y = 175.02x - 43.877$; $R^2 = 0.9999$); luteolin-6-*C*-glucoside ($y = 365.93x + 17.836$; $R^2 = 0.9997$); quercetin-3-*O*-glucoside ($y = 316.48x + 2.9142$; $R^2 = 1$), and quercetin-3-*O*-rutinoside ($y = 222.79x + 243.11$; $R^2 = 0.9998$). The results were expressed in mg per g of lyophilised decoction/infusion.

### 2.6. Statistical analysis

The results are expressed as mean value and mean standard deviation (SD). The results were analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with $\alpha = 0.05$.

This treatment was carried out using SPSS Version 16.0 (SPSS Inc., Chicago, IL).

## 3. Results

As far as we know, this is the first evaluation of the effects of drying (freeze or shade-drying) and preparation (oral dosage forms in water) methods on the antioxidant activity and phytochemical composition of wild and commercial samples of *T. lignosa* materials. For wild samples, shade-drying was performed simulating informants' general conditions of use and freeze-drying was carried out immediately after being collected; for both wild and commercial samples, decoctions and infusions were prepared according to folk recipes/formulations (Carvalho, 2010).

The antioxidant activity of the extracts obtained from the decoction/infusion preparations of wild (freeze or shade-dried) and commercial *T. lignosa* samples are shown in Table 1. The wild samples showed higher antioxidant activity than the commercial samples. However, the influence of the preparation procedure (decoction or infusion) on antioxidant activity was not clear. The infusion of the shade-dried wild sample showed the highest DPPH radical scavenging activity ($EC_{50}$ = 52 μg/mL) and reducing power ($EC_{50}$ = 21 μg/mL), while the decoction of the freeze-dried wild sample gave the highest lipid peroxidation inhibition in TBARS assay ($EC_{50}$ = 4 μg/mL). Both types of samples gave similar β-carotene bleaching inhibition ($EC_{50}$ = 12 μg/mL). It has to be highlighted that the studied samples revealed interesting antioxidant properties, particularly the infusion of shade-dried wild sample that gave higher reducing power than the standard Trolox. The antioxidant effects herein reported for *T. lignosa* support recommendations for its traditional use.

Ascorbic acid and free sugars were analysed and quantified (Table 2). Infusions gave higher amounts of ascorbic acid than decoctions, except in the freeze-dried wild sample. In fact, the decoction of this sample and the infusion of the shade-dried sample revealed higher ascorbic acid concentration (~5 mg/g) than the other samples. Regarding free sugars, it should be noted that the levels of the disaccharides sucrose and trehalose decreased in decoctions, being hydrolysed into fructose and glucose that increased in those samples in comparison with the corresponding infusions (Table 2). This phenomenon can also be observed in Fig. 1A for the shade-dried wild sample decoction that gave the highest fructose content (68 mg/g), while the infusion contained the highest level of sucrose (36 mg/g). The freeze-dried sample infusion showed the highest total sugars content (144 mg/g) and also the highest glucose concentration (49 mg/g). Fructose was the most abundant sugar in all the samples whatever their origin or preparation. The commercial sample (decoction or infusion) presented the lowest total sugars content (94–95 mg/g), despite the relatively high levels of trehalose found in its infusion (Table 2). Curiously, raffinose was not detected in the commercial sample (Fig. 1B).

Fig. 2 shows the chromatographic profile of the phenolic compounds detected in *T. lignosa* (exemplified for the infusion of the shade-dried sample), which includes mainly ellagitannins, followed by flavonoids (flavonols and flavones) and phenolic acid derivatives. Table 3 presents the data obtained from HPLC-DAD-MS analysis (retention time, $\lambda_{max}$ in the visible region, mass spectral data) used for the compound identification.

Ellagitannins were the major compounds found in the samples (peaks 1, 3, 4, 5 and 6). *T. lignosa* was already reported as a source of those compounds, which were suggested to be responsible for its antiviral activity against HIV (Bedoya et al., 2010). Peak 1 ($[M-H]^-$ at *m/z* 781) was assigned to punicalin (Seeram, Lee, Hardy, & Heber, 2005), whereas the rest of ellagitannins were associated with punicalagin derivatives. Peaks 3 and 5 ($[M-H]^-$ at *m/z* 1083) were identified as punicalagin isomers. Both compounds showed similar $MS^2$ fragmentation yielding ions at *m/z* 781, 601 and 301, corresponding to their cleavage to punicalin, gallagic acid and ellagic acid, respectively, as also reported by Seeram et al. (2005), and Fernández-Arroyo, Micol, Segura-Carretero, Fernández-Gutiérrez, and Barrajón-Catalán (2010). Peaks 4 and 6 ($[M-H]^-$ at *m/z* 1251) were tentatively identified as derivatives of punicalagin attached to gallic acid (punicalagin gallate isomers),

**Table 1**
Antioxidant activity ($EC_{50}$ values) of decoctions/infusions of different samples of *Tuberaria lignosa*.

| | Wild sample | | | | Commercial sample | | Standard |
|---|---|---|---|---|---|---|---|
| | Freeze-dried | | Shade-dried | | | | |
| | Decoction | Infusion | Decoction | Infusion | Decoction | Infusion | Trolox |
| Yield (%) | 21.2 ± 0.89 | 11.8 ± 0.44 | 19.7 ± 1.02 | 16.0 ± 0.27 | 20.8 ± 1.44 | 17.1 ± 0.11 | – |
| DPPH scavenging activity (μg/mL) | 57.3 ± 2.17$^{c}$ | 56.4 ± 0.89$^{c}$ | 58.7 ± 2.98$^{cb}$ | 52.1 ± 0.44$^{d}$ | 61.1 ± 0.95$^{b}$ | 65.9 ± 3.24$^{a}$ | 43.0 ± 1.71$^{e}$ |
| Reducing power (μg/mL) | 27.1 ± 0.78$^{d}$ | 30.4 ± 0.23$^{c}$ | 32.8 ± 0.55$^{b}$ | 21.3 ± 0.16$^{e}$ | 48.5 ± 0.57$^{a}$ | 32.1 ± 1.00$^{b}$ | 29.3 ± 3.15$^{c}$ |
| β-carotene bleaching inhibition (μg/mL) | 11.6 ± 2.55$^{d}$ | 15.1 ± 3.05$^{c}$ | 20.7 ± 1.60$^{b}$ | 11.7 ± 1.47$^{d}$ | 20.4 ± 1.55$^{b}$ | 25.8 ± 3.49$^{a}$ | 2.63 ± 0.14$^{e}$ |
| TBARS inhibition (μg/mL) | 3.99 ± 0.31$^{d}$ | 12.6 ± 0.49$^{b}$ | 10.1 ± 0.60$^{c}$ | 10.0 ± 0.49$^{c}$ | 19.2 ± 1.96$^{a}$ | 18.2 ± 0.11$^{a}$ | 3.73 ± 1.90$^{d}$ |

In each row different letters (a-e) mean significant differences ($p < 0.05$).

**Table 2**
Ascorbic acid and free sugars contents in the decoctions/infusions of different samples of *Tuberaria lignosa*.

| | Wild sample | | | | Commercial sample | |
|---|---|---|---|---|---|---|
| | Freeze-dried | | Shade-dried | | | |
| | Decoction | Infusion | Decoction | Infusion | Decoction | Infusion |
| Ascorbic acid (mg/g) | 4.75 ± 0.02$^{a}$ | 3.62 ± 0.01$^{d}$ | 4.47 ± 0.01$^{b}$ | 4.75 ± 0.01$^{a}$ | 3.27 ± 0.11$^{e}$ | 3.94 ± 0.03$^{c}$ |
| Fructose (mg/g) | 53.9 ± 0.76$^{b}$ | 53.4 ± 2.91$^{b}$ | 67.6 ± 1.93$^{a}$ | 53.1 ± 0.60$^{b}$ | 52.1 ± 1.70$^{b}$ | 45.56 ± 1.06$^{c}$ |
| Glucose (mg/g) | 43.7 ± 0.43$^{c}$ | 48.8 ± 1.30$^{a}$ | 45.8 ± 0.12$^{b}$ | 25.0 ± 0.36$^{e}$ | 30.6 ± 0.62$^{d}$ | 21.4 ± 0.12$^{f}$ |
| Sucrose (mg/g) | 7.72 ± 0.33$^{d}$ | 32.1 ± 1.55$^{b}$ | 11.2 ± 0.32$^{c}$ | 36.0 ± 0.91$^{a}$ | 3.58 ± 0.14$^{e}$ | 9.11 ± 0.43d$^{c}$ |
| Trehalose (mg/g) | 11.4 ± 0.09$^{c}$ | 12.1 ± 0.51$^{c}$ | 9.04 ± 0.74$^{d}$ | 13.7 ± 0.76$^{b}$ | 7.94 ± 0.00$^{d}$ | 19.2 ± 0.17$^{a}$ |
| Raffinose (mg/g) | 1.40 ± 0.10$^{a}$ | 2.37 ± 0.90$^{a}$ | 1.67 ± 0.05$^{a}$ | 2.51 ± 0.16$^{a}$ | nd | nd |
| Total sugars (mg/g) | 123 ± 1.92$^{c}$ | 144 ± 5.28$^{a}$ | 135 ± 3.05$^{b}$ | 130 ± 2.78$^{cb}$ | 94.2 ± 1.22$^{d}$ | 95.2 ± 1.44$^{d}$ |

nd – not detected. In each row different letters (a-f) mean significant differences ($p < 0.05$).

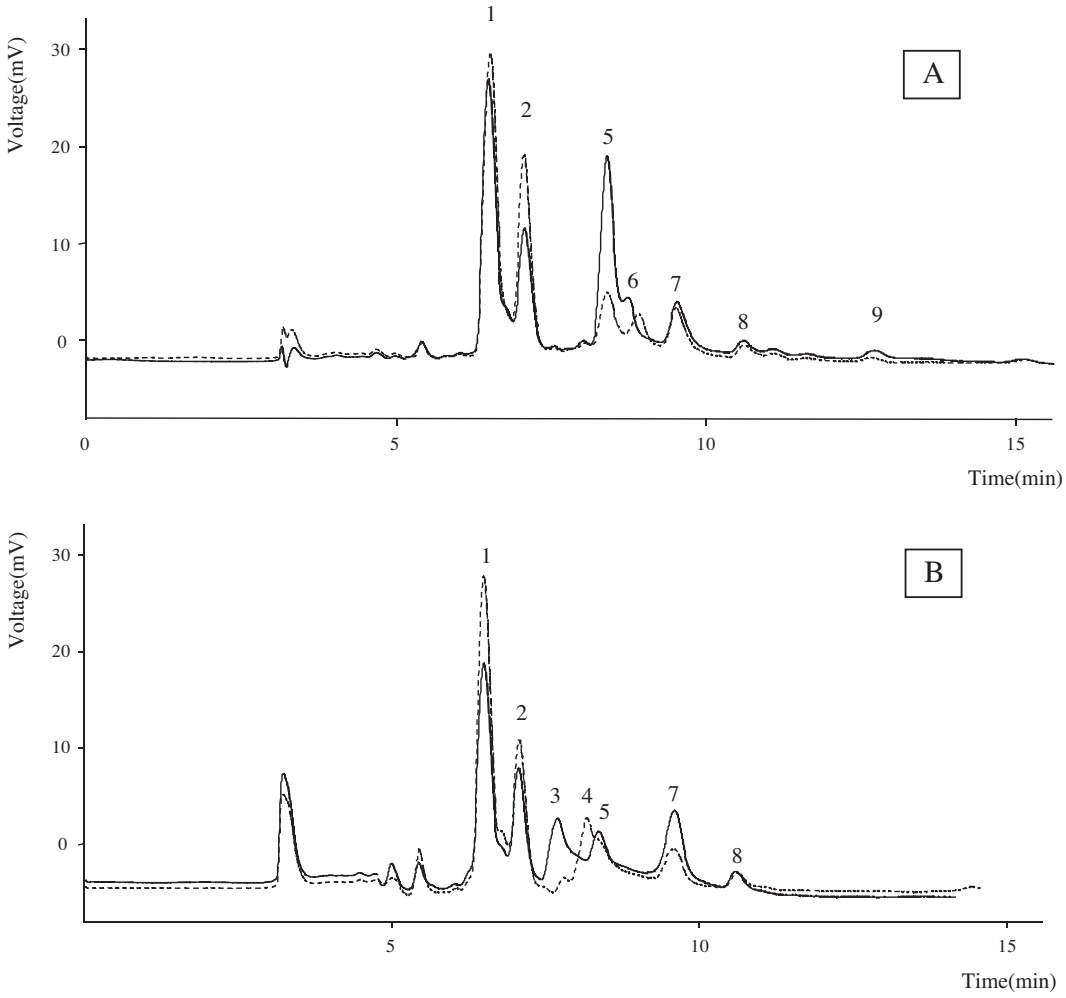


**Fig. 1.** (A) Free sugars in the infusion (–) and decoction (——) of *Tuberaria lignosa* shade-dried sample: 1-fructose; 2- glucose; 5-sucrose; 7-trehalose and 9-raffinose. (B) Free sugars in the infusion (——) and decoction (–) of commercial sample: 1-fructose; 2-glucose; 5-sucrose; 7-trehalose. Peaks 3, 4, 6 and 8 were not identified.

as previously reported by Saracini, Tattini, Traversi, Vincieri and Pinelli (2005). In these compounds, the gallic acid would not be bound to punicalagin by the carboxyl group through ester linkage, as denoted by the fragment at *m/z* 1083, corresponding to the loss of gallic acid itself.

Flavones and flavonols were also found in the studied samples. Peak 7 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 609 that released three $MS^2$ fragments ions at *m/z* 489 and 399, corresponding to losses of 120 and 90 amu, characteristic of *C*-hexosyl flavones, and at *m/z* 369 that might correspond to the luteolin aglycone bearing some sugar residues [luteolin + 83 amu] that remained attached to it (Ferreres, Llorach & Gil-Izquierdo, 2004; Ferreres, Silva, Andrade, Seabra & Ferreira, 2003). The fact that no relevant fragment derived from the loss of a complete hexosyl residue (−162 amu) was detected suggested that both sugars were *C*-attached, which allowed a tentative identification of the compound as luteolin-6-*C*-glucose-8-*C*-glucose. Peak 8 had a similar fragmentation pattern with a pseudomolecular ion $[M-H]^-$ at *m/z* 593, being tentatively identified as apigenin-6-*C*-glucose-8-*C*-glucose. Peaks 10 and 15 showed the same pseudomolecular ion $[M-H]^-$ at *m/z* 447 giving place to three $MS^2$ fragment ions, a major one at *m/z* 357 $[M-90]^-$, and another two at *m/z* 327 $[M-120]^-$ and at *m/z* 429 $[M-18]^-$. The fragmentation pattern was characteristic of *C*-glycosylated flavones at C-6/C-8, and the relative abundance of fragments pointed out to sugar substitution at C-8 (peak 10) and at C-6 (peak 15) according to the fragmentation patterns described by Ferreres et al. (2003) and Ferreres et al. (2004). These peaks were respectively identified as luteolin-8-*C*-glucoside and luteolin-6-*C*-glucoside; the identity of this latter was further confirmed by comparison with an authentic standard. Similarly, peaks 11 and 13, with the same pseudomolecular ion ($[M-H]^-$ at *m/z* 431) and similar $MS^2$ behaviour, were identified as apigenin-8-*C*-glucoside and apigenin-6-*C*-glucoside, this latter also confirmed by comparison with a standard.

Peaks 12 and 14 were identified respectively as quercetin 3-*O*-rutinoside (rutin) and kaempferol-3-*O*-rutinoside according to their retention time, UV spectra and mass characteristics compared with authentic standards. Peak 16 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 739, 146 amu greater than a kaempferol-rutinoside, which may correspond to either a rhamnosyl or a *p*-coumaroyl moiety. No good UV spectrum could be obtained for this peak so as to confirm or disprove the presence of a *p*-coumaroyl moiety (additional peak or shoulder around 310 nm). However, since the presence of such a hydroxycinnamoyl residue should be reflected in a greater loss of polarity and subsequent delay in its elution, the peak was tentatively identified as a kaempferol-*O*-rhamnoside-*O*-rutinoside.

Peak 17 presented a pseudomolecular ion at *m/z* 707 releasing two $MS^2$ fragments at *m/z* 593 ($[M-H-114]^-$, corresponding to a possible glutarate moiety) and at *m/z* 285 ($[M-H-114-308]^-$, loss of a possible rutinoside or *p*-coumaroylglucoside moiety). The UV

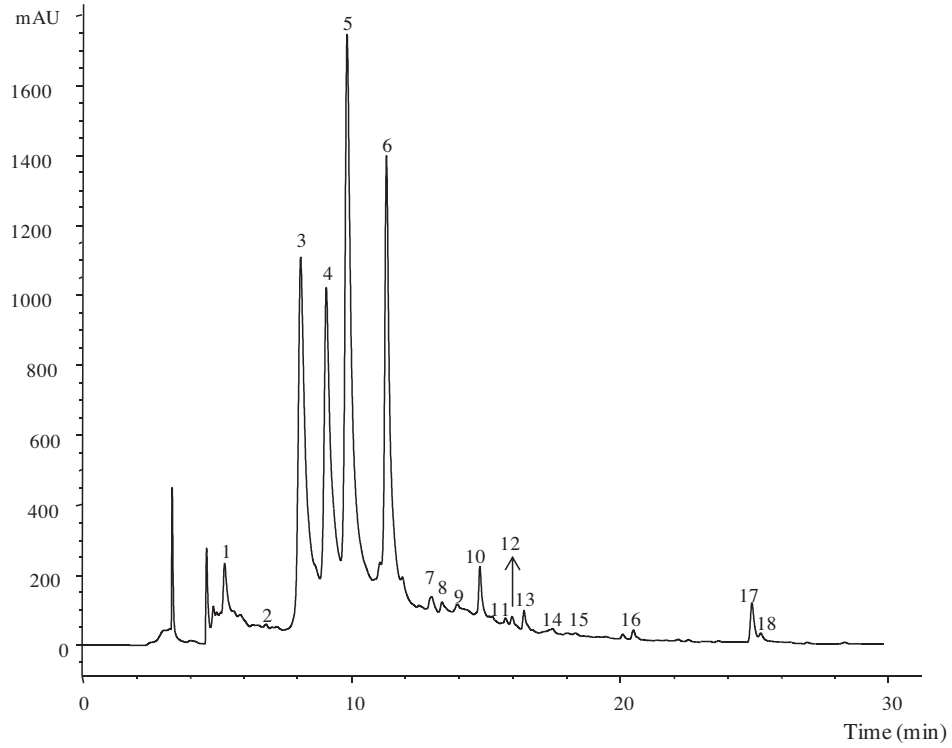


**Fig. 2.** HPLC profile of phenolic compounds in the infusion of *Tuberaria lignosa* shade-dried sample, recorded at 280 nm.

**Table 3**
Retention time (Rt), wavelengths of maximum absorption in the UV–vis region ($\lambda_{max}$), pseudomolecular and $MS^2$ fragment ions (in brackets, relative abundances) and tentative identification of the phenolic compounds in the decoctions/infusions of *Tuberaria lignosa*.

| Peak | Rt (min) | $\lambda_{max}$ (nm) | Molecular ion $[M-H]^-$ (*m/z*) | $MS^2$ (*m/z*) | Tentative identification |
|---|---|---|---|---|---|
| 1 | 5.2 | 268/378 | 781 | 781(100), 721(7), 601(20), 299(40) | Punicalin |
| 2 | 6.94 | 270 | 325 | 325(36), 169(100), 125(27) | Monogalloylglucose |
| 3 | 8.2 | 259/378 | 1083 | 1083(100), 781(6), 601(14), 301(4) | Punicalagin isomer 1 |
| 4 | 9.2 | 258/378 | 1251 | 1251(72), 1083(23), 781(3), 601(10), 301(22) | Punicalagin gallate isomer 1 |
| 5 | 9.98 | 258/378 | 1083 | 1083(100), 781*, 601(12), 301(10) | Punicalagin isomer 2 |
| 6 | 11.42 | 264/378 | 1251 | 1251(60), 1083(11), 781(6), 601(40), 301(51) | Punicalagin gallate isomer 2 |
| 7 | 12.03 | 350 | 609 | 609(100), 489(33), 399(9), 369(16) | Luteolin-6-*C*-glucose-8-*C*-glucose |
| 8 | 13.03 | 334 | 593 | 593(100), 473(13), 383(6), 353(21) | Apigenin-6-*C*-glucose-8-*C*-glucose |
| 9 | 14.07 | 310 | 337 | 191(100), 173(16), 163(6) | 5-*O*-*p*-Coumaroylquinic acid |
| 10 | 14.92 | 350 | 447 | 429(7), 357(60), 327(100), 285(8) | Luteolin-8-*C*-glucoside |
| 11 | 15.40 | 356 | 431 | 413(4), 341(37), 311(100) | Apigenin-8-*C*-glucoside |
| 12 | 16.11 | 354 | 609 | 301(100) | Quercetin-3-*O*-rutinoside |
| 13 | 16.58 | 334 | 431 | 413(8), 341(60), 311(100) | Apigenin-6-*C*-glucoside |
| 14 | 17.58 | 348 | 593 | 285(100) | Kaempferol-3-*O*-rutinoside |
| 15 | 18.57 | 350 | 447 | 357(4), 327(17), 285(24) | Luteolin-6-*C*-glucoside |
| 16 | 20.93 | – | 739 | 593(100), 285(11) | Kaempferol-*O*-rhamnoside-*O*-rutinoside |
| 17 | 25.20 | 314,354sh | 707 | 593(100), 285(14) | Kaempferol-*p*-coumaroylglucoside-glutarate |
| 18 | 25.52 | 314,354sh | 593 | 447(10), 285(69) | Kaempferol-*p*-coumaroylglucoside |

* Relative abundances < 1.

spectrum of the peak was not characteristic of a kaempferol-rutinoside as it presents maximum wavelength at 314 nm, which could confirm the presence of a *p*-coumaroyl residue, so that the peak could be tentatively identified as kaempferol-*p*-coumaroylglucoside-glutarate. Peak 18 showed similar characteristics to peak 14, but it released an additional minor $MS^2$ fragment at *m/z* 447 (loss of 146 amu that may correspond to either a rhamnosyl or a *p*-coumaroyl moiety). Its delayed retention time and UV spectrum suggested that it may be kaempferol-*p*-coumaroylglucoside.

Finally, peaks 2 and 9 were associated to phenolic acid derivatives. The characteristics of peak 2, with a pseudomolecular ion $[M-H]^-$ at *m/z* 331 and a majority $MS^2$ fragment at *m/z* 169 ($[M-162]^-$, loss of a hexosyl moiety) consistent with gallic acid, allowed its assignment as a monogalloylglucose. Peak 9 ($[M-H]^-$ at *m/z* 337) was identified as 5-*O*-*p*-coumaroylquinic acid according to its $MS^2$ fragmentation pattern as reported by Clifford, Johnston, Knight, & Kuhnert, 2003, and Clifford, Knight, and Kuhnert (2006) for caffeoylquinic acid isomers.

As for the other analysed compounds (ascorbic acid and sugars), the type of sample preparation did not show a clear influence on the composition of the extracts (Table 4). Little differences were found among the concentrations and distribution of phenolics in the different extracts (decoction or infusion) obtained from the two types of wild *Tuberaria* samples (shade- or freeze-dried). However, much lower amounts of phenolic compounds, namely ellagitannins, were found in the preparations of the commercial sample.

**Table 4**
Quantification of the phenolic compounds in the decoctions/infusions of the different samples of *Tuberaria lignosa*.

| | Wild sample | | | | Commercial sample | |
|---|---|---|---|---|---|---|
| | Freeze-dried | | Shade-dried | | | |
| Peak | Decoction | Infusion | Decoction | Infusion | Decoction | Infusion |
| 1 | tr | tr | 1.24 ± 0.03 | tr | 11.72 ± 0.94 | 11.22 ± 0.13 |
| 2 | tr | tr | tr | tr | tr | tr |
| 3 | 44.7 ± 2.26 | 43.3 ± 0.94 | 46.2 ± 1.29 | 51.2 ± 0.27 | 39.6 ± 0.61 | 38.0 ± 0.57 |
| 4 | 51.8 ± 2.82 | 49.9 ± 0.03 | 46.3 ± 0.04 | 50.0 ± 0.87 | 7.27 ± 0.44 | 5.20 ± 0.15 |
| 5 | 78.2 ± 0.91 | 79.4 ± 1.50 | 78.5 ± 1.29 | 89.6 ± 1.10 | 67.8 ± 0.12 | 64.1 ± 1.23 |
| 6 | 46.5 ± 1.53 | 44.9 ± 2.10 | 40.5 ± 1.98 | 41.9 ± 0.50 | 7.79 ± 0.12 | 6.81 ± 0.63 |
| 7 | 0.68 ± 0.03 | 1.03 ± 0.06 | 0.71 ± 0.01 | 0.69 ± 0.01 | 0.84 ± 0.11 | 0.79 ± 0.09 |
| 8 | 1.63 ± 0.03 | 1.94 ± 0.49 | 1.81 ± 0.02 | 1.73 ± 0.11 | 2.01 ± 0.25 | 1.87 ± 0.11 |
| 9 | 0.38 ± 0.08 | 0.53 ± 0.06 | 0.34 ± 0.01 | 0.36 ± 0.06 | 0.84 ± 0.12 | 0.93 ± 0.02 |
| 10 | 5.26 ± 0.26 | 3.86 ± 0.36 | 4.92 ± 0.12 | 4.20 ± 0.03 | 2.18 ± 0.18 | 2.09 ± 0.19 |
| 11 | 1.47 ± 0.01 | 1.56 ± 0.04 | 1.55 ± 0.00 | 1.55 ± 0.02 | 1.65 ± 0.02 | 1.57 ± 0.04 |
| 12 | 0.79 ± 0.02 | 0.60 ± 0.02 | 0.68 ± 0.02 | 0.70 ± 0.02 | 0.53 ± 0.04 | 0.54 ± 0.05 |
| 13 | 2.47 ± 0.07 | 2.39 ± 0.01 | 2.40 ± 0.01 | 2.15 ± 0.00 | 1.81 ± 0.09 | 1.80 ± 0.07 |
| 14 | 0.77 ± 0.02 | 0.40 ± 0.02 | 0.51 ± 0.02 | 0.46 ± 0.00 | 0.72 ± 0.06 | 0.80 ± 0.03 |
| 15 | 0.05 ± 0.00 | 0.03 ± 0.00 | 0.06 ± 0.00 | 0.04 ± 0.01 | 0.21 ± 0.01 | 0.20 ± 0.01 |
| 16 | 0.33 ± 0.02 | 0.32 ± 0.00 | 0.28 ± 0.00 | 0.27 ± 0.02 | 0.24 ± 0.01 | 0.31 ± 0.02 |
| 17 | 4.54 ± 0.08 | 3.78 ± 0.23 | 4.12 ± 0.05 | 3.79 ± 0.04 | 4.80 ± 0.09 | 4.18 ± 0.43 |
| 18 | 1.49 ± 0.03 | 1.35 ± 0.08 | 1.26 ± 0.05 | 1.37 ± 0.02 | 1.16 ± 0.07 | 1.42 ± 0.15 |
| **TPA** (mg/g) | $0.38 \pm 0.08^{c}$ | $0.53 \pm 0.06^{b}$ | $0.34 \pm 0.01^{c}$ | $0.36 \pm 0.06^{c}$ | $0.84 \pm 0.12^{a}$ | $0.93 \pm 0.02^{a}$ |
| **TEAD** (mg/g) | $221 \pm 7.51^{ba}$ | $218 \pm 4.65^{b}$ | $213 \pm 4.63^{b}$ | $233 \pm 0.47^{a}$ | $134 \pm 0.13^{c}$ | $125 \pm 0.90^{c}$ |
| **TF** (mg/g) | $19.5 \pm 0.50^{a}$ | $17.3 \pm 0.19^{cb}$ | $18.3 \pm 0.01^{b}$ | $17.0 \pm 0.13^{c}$ | $16.2 \pm 0.81^{cd}$ | $15.6 \pm 1.11^{d}$ |
| **TP** (mg/g) | $241 \pm 7.97^{ba}$ | $235 \pm 4.65^{b}$ | $232 \pm 4.64^{b}$ | $250 \pm 0.57^{a}$ | $151 \pm 0.74^{c}$ | $142 \pm 2.13^{c}$ |

tr – traces; TPA – total phenolic acid derivatives; TEAD– total ellagic acid derivatives; TF – total flavonoids; TP – total phenolic compounds. In each row different letters (a-d) mean significant differences ($p < 0.05$).

Punicalagin derivatives were the most abundant compounds in all the samples.

## 4. Discussion

In general, the antioxidants (phenolic compounds, vitamins, etc.) content of fresh plant materials is higher than that of dried plant materials, due to their degradation during drying. However, some recent studies have shown that dried plant materials (including air-drying) contain higher antioxidants, such as polyphenolics, and antioxidant activity as compared to fresh plant materials (Suvarnakuta, Chaweerungrat, & Devahastin, 2011). Medicinal plants are often dried and stored for a long time before use in manufacturing various types of products (Lin, Sung, & Chen, 2011). Dehydration is an important preservation method for plant material, as it inhibits enzymatic degradation and limits microbial growth of plants (Harbourne, Marete, Jacquier, & O'Riordan, 2009; Muller & Heinds, 2006, chap. 17). Drying can be performed using different methods. Traditionally, in folk medicine, medicinal plants are dried in the shade (Sellami et al., 2011). Freeze-drying is generally better to preserve the quality of medicinal plants during processing (Abascal, Ganora, & Yarnell, 2005; Pinela et al., 2011). The length of storage period also has a significant influence on the quality of medicinal plants, reducing the antioxidant activity and antioxidants content (Guimarães, Barreira, Barros, Carvalho & Ferreira, 2011).

In the present work, the influence of the drying process applied to wild samples was not so evident as previously observed by us and by others in other plant species, where freeze-drying proved to preserve more the composition, including phenolics, ascorbic acid, sugars, tocopherols, chlorophylls, and lycopene contents, as well as antioxidant activity (Chan et al., 2009; Pinela et al., 2011). Nevertheless, Hossain, Barry-Ryan, Martin-Diana, and Brunton (2010) reported that air-dried samples had significantly higher antioxidants content and antioxidant capacity than freeze-dried and vacuum oven-dried samples. Fresh samples had the lowest values for the parameters tested. Oven-drying resulted in higher parameters than freeze-drying. As stated, the effects of drying methods on antioxidants and antioxidant activity are not conclusive. In fact, in the performed study, the effects of drying process were not evident and, as it can be observed in Tables 2 and 4, the magnitude of concentrations of the different compounds in wild samples is similar. This absence of marked effects of drying methods was also reported by Hsu, Chen, Weng, and Tseng (2003).

Regarding the influence of preparation method (decoction/infusion), the expected higher degradation of compounds in decoctions was not always observed. This could be due to the different amounts of sample used to prepare decoctions and infusions. To perform the present study, it was decided to use the amounts exactly indicated by local informants in order to mimic the traditional use of *T. lignosa*. Therefore, a higher amount was used in decoctions preparation, leading to a higher extraction yield (Table 1) that probably compensated for the loss of compounds. It should be highlighted that consumers using higher amounts of plant material for decoctions than for infusions, empirically know what was proved experimentally herein.

The most relevant effects were observed between wild and commercial samples that gave completely different amounts of antioxidants and antioxidant activity. The loss of phytochemical properties observed in commercial samples could be linked to processing (drying, preserving and packing) and storage time, since the vegetal material of wild and commercial sample could came from the same localities, although gathered separately in different periods.

## 5. Conclusions

Freeze-dried wild sample (infusion) gave the highest levels of sugars, while shade-dried wild sample (infusion) and freeze-dried sample (decoction) presented higher ascorbic acid and phenolic compounds (including ellagic acid derivatives and flavonoids) content than the other samples. The last two mentioned samples also revealed higher antioxidant activity than the other samples and, in some cases, even higher than Trolox. Decoctions gave lower amounts of disaccharides than infusions, which seemed to be hydrolysed, increasing the content in monosaccharides. Commer-

cial samples showed the lowest contents in all the analysed compounds, and especially in ellagitannins and flavonoids, which was also consistent with their lower antioxidant activity in all the assays compared with the wild samples.

As far as we know this is the first detailed characterisation of the antioxidant properties and phytochemical composition of wild and commercial samples of *T. lignosa*. The obtained results give scientific and technical support to the traditional uses of the wild plant in folk medicine, highlighting its decoctions and infusions as a source of bioactive compounds (e.g. phenolic compounds and ascorbic acid) to be used as functional beverages. Further studies should be performed in order to understand the mechanism of action of the mentioned compounds.

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